MODIFIED BARLEY α-GLUCOSIDASE

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US provisional application number 60/260,787 filed January 10, 2001.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] To be determined.

BACKGROUND OF THE INVENTION

[0003] In the germination of seeds of cereal plants, starch degradation is an important metabolic process. Starch is the primary source of carbon and energy for cereal seedlings until they become autotrophic. Degradation of cereal starches in cereal seedlings is a result of the concerted action of several enzymes including α-amylase, β-amylase, debranching enzyme and α-glucosidase. It has been observed that during the early stages of starch hydrolysis in germinating cereal seeds, α-amylase is the most important enzyme and α-glucosidase is the second most important enzyme to the seedlings starch degradation processes.

[0004] Starch degradation processes are important for other reasons besides the viability and vigor of cereal seedlings. Many food processes involve the conversion of starch from cereal plants for food or other uses. It is known that α-glucosidase accelerates the initial hydrolysis of starch granules in the presence of α-amylase. In vitro, barley α-glucosidase can hydrolyze native starch granules at a rate comparable to α-amylase. In addition, the two enzymes act synergistically in the starch degradation process.

[0005] For food production applications, and in other industrial processes to produce or process starches from cereals, thermal stability of enzymes becomes an important criteria. For example, the thermal stability of α-glucosidase is important because the conversion of barley starch to fermentable sugars during the industrial production of ethanol, as in brewing or in fuel ethanol production, typically takes place at temperatures of 65 to 73°C. The thermal lability of many native barley α-glucosidase enzymes results in either reduced efficiency of starch break down at the higher temperatures used for starch gelatinization, or requires that the starch be cooled to a more favorable temperature for enzymatic hydrolysis after the starch is gelatinized.

[0006] Significant research has occurred on barley α-glucosidase in the last few years. In fact, the native barley gene for α-glucosidase has been sequenced, cloned, and the amino acid

sequence of the resulting expressed enzyme has been determined. The DNA sequence of the native cDNA and the amino acid sequence of the protein are fully described in US Patent Number 5,763,252, the disclosure of which is incorporated herein by reference.

[0007] While the full sequence of barley α-glucosidase is known, many critical details about the structure and function of the enzyme are still uncharacterized. No crystal structure has been determined for any α-glucosidase of the glucosyl hydrolase family, making it much more difficult to intelligently select targets for mutagenesis. It is known that the α-glucosidase genes from various plants do have variations in their thermostability, but the rationales and reasons behind those differences are obscure. The lack of thermostable α-glucosidases has been a limitation in the industrial use of α-glucosidase enzymes to replace or supplement α-amylases in industrial hydrolysis systems. Thus the need exists for more thermostable α-glucosidases which can be used for a wide variety of industrial and food preparation purposes such as specifically brewing and fuel ethanol production.

BRIEF SUMMARY OF THE INVENTION

[0008] The present invention is summarized in that a barley α-glucosidase has been subjected to conservative mutation to create variants of the amino acid sequence of the native enzyme which are more thermostable and therefore more suitable for industrial purposes.

[0009] It is an object of the present invention to provide for the creation of mutant forms of barley α-glucosidase genes which encode enzymes having increased thermostability for incorporation in transgenic barley plants which are thereby more suitable for industrial utilization in processes requiring starch hydrolysis.

[00010] Other objects, advantages, and features of the present invention will become apparent from the following specification when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[00011] Fig. 1 is a graphical illustration of the thermostability of various plant α-glucosidase enzymes.

[00012] Fig. 2 is a sequence alignment of the amino acid sequence of various plant α-glucosidases.

[00013] Figs. 3, 4 and 5 are graphical illustration of some of the experimental results from the results of the examples below.

DETAILED DESCRIPTION OF THE INVENTION

[00014] In order to consider directed experiments to make a barley α-glucosidase that has improved thermostability, the first problem is the lack of information. By function, the α-glucosidase enzyme is classified as a member of the glycosyl hydrolase 31 family of enzymes. Not only is there no known three-dimensional study of the structure of the α-glucosidase enzyme, there is no known crystal structure for any member of that family of enzymes. It is therefore not possible to directly study the tertiary structure of the enzyme to identify what locations might be suitable to consider for making changes to the molecule to add thermal stability. Accordingly, indirect methods were used to identify sites of directed mutagenesis where potential changes in amino acids would add to the thermal characteristics of the enzyme. To approach that question, the available information about plant α-glucosidases was examined. The literature contains the sequences of several known α-glucosidase genes from various plants. It was also known that the enzymes encoded by those genes contain significant variation in their thermostability. Fig. 1 illustrates the variation in thermostability among several known α-glucosidase isoforms. In Fig. 1, the results obtained with the α-glucosidase from barley are indicated by the closed circles, the sugar beet enzyme results are charted by the open circles, the characteristics of the spinach enzyme are shown by the closed triangle and the thermal characteristics of the Arabidopsis enzyme are illustrated by the open triangle. Each enzyme is compared to a non-heated control sample of the same enzyme. This data suggests that the barley enzyme is one of the less thermostable of known plant α-glucosidase isoforms and that it should be possible to improve its thermal characteristics. A project thus was initiated to make directed sequence modifications to the amino acid sequence of the barley α-glucosidase enzyme, the locations of the modifications being selected based on sequence comparisons to other plant α-glucosidase genes. In essence, the idea is to test modifications to the barley gene to incorporate into the enzyme amino acid residues found in other plant α-glucosidases.

[00015] To facilitate this process, an alignment study of the various known plant α-glucosidase genes was conducted. This alignment is represented in an alignment table, shown in Fig. 2. Fig. 2 shows the best-fit alignment of the amino acid sequence of the α-glucosidase genes from barley, sugar beet, spinach and Arabidopsis, using the conventional single letter representations for the amino acids. Capital letters indicate identity to the barley sequence. This sequence comparison information can be combined with information about predicted secondary

structure of the protein, available from computer analysis of the sequence, to begin to identify sites for mutation to create better thermostability.

[00016] The data presented above demonstrated that the differences in thermostability among the plant α-glucosidase enzymes was significant. For example, the enzyme from sugar beet still retains 60% of its maximal activity following exposure to 75°C for 10 minutes. By contrast, the native form of barley α-glucosidase retains only 10% of its maximal activity after exposure to 55°C for 10 minutes. The spinach and Arabidopsis enzymes are between these two extremes, with the spinach enzyme being the second highest in thermostability and the Arabidopsis enzyme third. Analysis of the deduced amino acid sequences among the four enzymes showed that the barley sequence actually had a relatively high level of sequence identity with the sugar beet and spinach enzymes, 50.8% and 53.6%, respectively. Thus the differences which do exist between the barley sequence and those of sugar beet and spinach likely account for the differences in thermal behavior. So a possible approach to adding thermal stability to the barley enzyme is to make it more like the homologous enzymes from other plants. The problem then became deciding which changes to the barley sequence would have the desired effect.

[00017] One set of differences in amino acid sequence which was identified is that the sugar beet, spinach and Arabidopsis sequences had four commonly conserved proline residues not found in the barley enzyme. These residues were prolines at position 336, 340, 547 and 742 (based on the homologous position in the barley sequence). It is also known that proline residues can be important for thermostability (Suzuki, Y., Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 65: 146-148(1989)). Thus it was decided to test directed mutations of the barley sequence to add proline residues at positions 336, 340 and 742 to test this possibility. No mutation was attempted at position 547 because the secondary structure predictions made using the program Peptide Structure (Wisconsin GCG Package, Madison, WI) predicted that this residue is in the middle of a β-sheet. It has been reported that the addition of a proline to a β-sheet would not enhance thermostability of an enzyme (Watanabe, et al., Y. Eur. J. Biochem. 226: 2777-283 (1994)).

[00018] Based on this analysis, genes encoding mutant forms of the barley α-glucosidase enzyme were created and tested for thermostability. The native barley sequence was available, as disclosed in U.S. Patent No. 5,763,252. Conservative changes were engineered into the native barley DNA sequence to change a single codon to code for proline in substitution for another amino acid. The genes encoding the enzymes were cloned into suitable expression vectors and expressed in yeast. The mutant enzymatic forms designated T340P (this nomenclature indicating a "T" or threonine residue at location 340 in the native sequence has been changed to a "P" or

proline residue) and A742P (alanine to proline at residue 742) exhibited activity, but a similar mutation at position 336 (R336P – arginine to proline) failed to yield a protein with appropriate detectable enzymatic activity.

[00019] All three mutated enzymes were tested for thermal performance compared to the native barley enzyme. The mutant form T340P was tested first at pH 6.0. The temperature at which the wild-type enzyme showed only 50% of its activity was 48°C. By contrast the mutant enzyme isoform T340P did not show any decrease in activity until after it was heated to 50°C, and the temperature at which 50% of the activity was lost was found to be 58°C, which represents an improvement of 10°C from the wild type. Since thermostability may be decreased in lower levels of pH, the test was repeated at a pH of 4.0, at which the temperature for a loss of 50% activity for the wild type was 36°C and for the T340P was 43°C. A similar test at pH 6.0 was conducted with A742P, but that test did not yield an improved result for this mutant isoform at this pH.

[00020] Based on this observation, the protein sequence was studied for other possible modifications which might improve thermal stability of the wild type barley α-glucosidase. The modifications considered were those which would add prolines, remove or add glycosylation sites, or remove possible sites of deamidation or hydrolysis of peptide bonds at aspartic acid residues. Proline residues can be added because several studies show an increase in thermostability due to the addition of prolines at key sites. The mechanism of proline stabilization revolves around the presence of proline residues at the second sites of β-turns and in the first turn of α-helices (Watanabe et al., FEBS Lett. 290:221-223 (1991)). Chen et al., Protein Eng. 8:575-582(1994) showed that the thermostability of fungal glucoamylase can be increased by decreasing the deamidation of selected asparagine-glycine sequences by substituting alanine for asparagines. Ahern and Klibinov, Science 228:1280-1284 (1985) found that the thermal inactivation of lysozyme can attributed to both the deamidation of asparagine-glycine sequences as well as the hydrolysis of aspartate-X peptide bonds. Therefore, it is envisioned that aspartic acid residues will be changed to glutamic acid residues. There is evidence that both the addition, Olsen and Thomsen, J. of General Microb. 137: 579-585 (1991), and removal, Meldgaard and Svendsen, Microbiology 140:159-1661994, of N-glycosylation has shown an increase in thermostability in various enzymes. Therefore, N-glycosylation sites will either be introduced or removed using site-directed mutagenesis.

[00021] What follows as Table 1 is a list of proposed additional mutations designed to enhance the thermostability of barley alpha-glucosidase based on the sequence alignment

between the barley enzyme and the sugar beet, spinach, and Arabidopsis enzymes. These mutants will remove either deamidation sites or aspartic acids, add or remove N-glycosylation sites, or add prolines. The influence of prolines on nearby residues is so strong that the imprudent substitution of proline for another amino acid might result in the destruction of both secondary and tertiary structures, as well as the loss of protein function and stability. Therefore, prolines will only be substituted if the residue would be in the first turn of an α-helix or second site of a β-turn. Since there is no crystal structure for barley α-glucosidase the presumed presence of α-helices and β-turns is based on the secondary structure predictions using computer analysis.

TABLE 1

D83E	Removing an aspartate
D92E	Removing an aspartate
G100P	Adding a proline
D101P	Adding a proline, Removing an aspartate
D105E	Removing an aspartate
A122P	Adding a proline
S184P	Adding a proline
N298D	Removing N-glycosylation site
R336P	Adding a proline
D369E	Removing an aspartate
D372N	Adding N-glycosylation site, removing an aspartate
N391D	Removing N-glycosylation site
N394P	Adding a proline
D403P	Adding a proline, removing an aspartate
D463S	Adding N-glycosylation site
D508E	Removing an aspartate
N568A	Removing a deamidation site
D694N	Adding N-glycosylation site, removing an aspartate
A713P	Adding a proline
A742P	Adding a proline
D764E	Removing an aspartate

[00022] Since the above modifications to the native barley α-glucosidase enzyme are made without support from the analysis of the tertiary structure of the protein, some of these changes might result in decrease or absence of enzymatic activity or a change in thermal characteristics in an unwanted direction. Accordingly, each of these proposed alterations should be separately tested, as described below with the T340P mutant form. However, the data presented here demonstrates that this strategy can be successfully implemented. Since the method for selecting sites for directed mutagenesis are presented here, and since a method for testing the thermal stability is also described, it is now possible for those of skill in the field to test the mutations proposed above to determine empirically which ones add to the thermostability of the enzyme.

[00023] Techniques for site directed mutagenesis of DNA modifications are well known to those of skill in the art. In short, segments of constructed DNA of specific sequence can be substituted for segments of DNA from the native coding sequence to produce any sequence desired. It has also become common in the field to take mutant forms of coding sequences encoding proteins and clone those sequences into widely available expression vectors to express the coding sequences in a host, which can be heterologous to the native gene or not. Since protein production mechanisms are generally conserved within eukaryotic organisms, such a mutant protein can be most conveniently produced for testing its properties in whatever convenient eukaryotic expression system is available, including both host based systems, such as yeast, as well as systems based on cell-free gene expression.

[00024] It is also understood that because of the degeneracy of the genetic code, many different DNA sequences can encode the same protein. Hence, many changes to DNA sequences are possible without changing the protein produced from expression of a coding sequence. Also, it is possible to make modest conservative changes to the amino acid sequence of a protein without changing its functionality or characteristics in any significant manner. Such minor changes are within the scope of the invention claimed here. This document also contains DNA and/or protein sequences. While these sequences are believed correct, given the limits of present technology, it is possible that there might be one or more small errors, whether by insertion, substitution or deletion. However, since the sequences are certainly almost completely correct, those of skill in the art know how to work around and correct minor sequence errors of this type.

EXAMPLES

[00025] Chemicals and reagents. Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated.

[00026] Plant sources. Seeds of a sugar beet breeding line (ACS9400461) were kindly provided by Professor I. Goldman (University of Wisconsin). Arabidopsis (v. Columbia) seedlings were grown under a 10 hour photoperiod at a temperature of 25 °C for 3 weeks before harvesting. Spinach seeds (cv. Bloomsdale Longstanding, Northrup King) were purchased locally. Barley seeds (cv. Morex) were imbibed, germinated and kilned as described by Henson and Stone, J. Chromatog. 469:361-367 (1989).

[00027] Isolation of crude extracts from plants. Crude extracts from malted barley, seeds of sugar beet and spinach, and leaves from Arabidopsis were isolated using published protocols (Im and Henson, Carbohydr. Res. 277:145-159 (1995); Chiba et al., Agric. Biol. Chem. 42: 241-245 (1978); Sugimoto et al., L. Biosci. Biotech. Biochem. 59: 673-677 (1995); Monroe et al., Plant Physiol. 119: 385-397 (1999)). The extracts were dialyzed (16 hours, 4 °C) against 50 mM sodium-succinate, pH 4.5.

[00028] Enzyme assay. α-Glucosidase activities were measured by the release of glucose from maltose. Unless otherwise stated, the enzyme was incubated for 1 hour at 30 °C with 25 mM maltose in 50 mM sodium-succinate (pH 4.5) during which time substrate hydrolysis rates were linear. The glucose released was quantified by determining the reduction of NAD by the coupled reactions of hexokinase and glucose-6-dehydrogenase (Im and Henson, Carbohydr. Res. 277:145-159 (1995)).

[00029] Thermostability testing of plant extracts. Enzyme extracts were incubated for 10 minutes at temperatures ranging from 5 to 75 °C. The residual rate of maltose hydrolysis was assayed for 1 hour at 30 °C.

[00030] Alignment of α-glucosidase sequences from four plant species. Alignment of the published α-glucosidase amino acid sequences from barley (Genbank accession number U22450), spinach (D86624), sugarbeet (D89615), and Arabidopsis (AF014806) was done using the program Align Plus-Version 2.0 (Scientific and Educational Software). The results of the alignment are shown on Fig. 2.

[00031] Mutagenesis. Mutagenesis was done using the Muta-Gene kit (BIO-RAD). Barley α-glucosidase cDNA was sub-cloned into the EcoRI site of the phagemid pTZ18U (BIO-RAD, Hercules, CA). E. coli strain CJ236 (Kunkel et al., 1987) was used to generate dU-substituted DNA and single stranded DNA was isolated using the helper phage M13K07 (BIO-RAD). For the mutant R336P, the oligonucleotide CGGTGAAGTTGACAGGATCCAAGGTGAAG (5', reverse complement) was used to replace the codon for arginine (CGT) with a codon for proline (CCT) and to remove a Tth111I site. For the mutant T340P, the oligonucleotide GAGCTCGGCGGCGGGGAAGTTTACACGGTC was used to replace the codon for threonine (ACC) with a codon for proline (CCC) and to remove a Tth111I site. For the mutant A742P, the oligonucleotide CCAGGAGGTGGAACGGGGTCCGGCGC was used to replace the codon for alanine (GCG) with a codon for proline (CCG) and to remove a RsrII site.

[00032] Sequencing. The mutated cDNA was sequenced using the Sanger method with an automatic sequencer by the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL.

[00033] Expression. The mutated cDNA was subcloned into the EcoRI sites of the Pichia pastoris vector pPIC9K (Invitrogen) and transformed into P. pastoris GS115 using the Pichia EasyComp kit (Invitrogen). Ten histidine autotrophs (His+) were induced with methanol following the instructions in the Pichia Expression Kit (Invitrogen). Pichia colonies that secreted measurable α-glucosidase activity were used for thermostability studies.

[00034] Thermostability testing of wild-type and mutated α-glucosidase. Enzyme extracts from non-mutated, recombinant α-glucosidase (rAGL), T340P and A742P were incubated for 10 minutes at temperatures ranging from 0 to 60 °C at a pH of either 6.0 or 4.0. The residual rate of maltose hydrolysis was assayed for 18 hours at 30 °C at pH 4.5.

[00035] Results. Fig. 3 illustrates the improved thermostability of the T340P mutant form of the enzyme as compared to wild type barley α-glucosidase. In Fig. 3, the thermostability of the native barley α-glucosidase (squares) is compared that of the T340P enzyme (triangles) at a temperature range of up to 60°C at a pH of 6.0. Activity is compared to the same enzyme extract unheated. Fig. 4 shows a similar test at a pH of 4.0, again with the native form indicated by squares and the T340P indicated by triangles. Fig. 5 shows an Arrhenius plot of the native and

the T340P. Enzyme extracts of recombinantly produced wild-type and the T340P were incubated at temperatures from 0 to 55°C, and assayed at the same temperatures at pH of 4.5. The results are plotted as log V_i vs. $10^{-3}/T(°C)$.

[00036] Mashing. The effect of the thermostable modified barley α-glucosidase was then tested in mashing. Mashing is the process whereby a nutrient solution capable of supporting fermentation by brewer's yeast is made from malted barley. The sugars that yeast ferment are glucose, maltose and maltriose. Mashing was conducted in the presence of non-modified recombinant barley α-glucosidase (designated rAGLwt, control) and in the presence of the modified T340P barley α-glucosidase. The amount of glucose produced by the mashes containing the T340P enzyme was found to be 29% greater than that produced in the control mashes. Similarly, there was 25% more maltose and 26% more maltriose in the mashes containing T340P than in mashes containing the rAGLwt. In addition, the real degree of fermentation values (RDF), calculated on a per unit α-glucosidase added to the mash, were higher in the mashes with added T340P. The mashes containg the T340P also had higher concentrations of maltotetraose, maltopentaose, maltohexaose, and maltoheptaose, but the differences may not have been statistically significant. The new results of the mashing processes demonstrated that the modified barley α-glucosidase increases the amount of fermentable sugars in real world processes, and thus provides new options for brewers to obtain desirable carbohydrate profiles in brewed products.

[00037] Transgenic plants. The gene encoding T340P can also be expressed in transgenic plants. Plant gene expression cassettes are widely available based on strong constitutive promoters, and condition and tissue specific promoters are now becoming available. The coding sequence for the T340P enzyme can be placed into such an expression cassette and transformed into barley, which is susceptible to particle mediated plant transformation techniques. Since the improvement in thermal stability in the T340P enzyme is due to the sequence modification, the enzyme expressed in transgenic barley will have the same thermal processing characteristics as the enzyme produced in the yeast described above.

[00038] Other modifications. The modified barley α-glucosidase isoforms N298D, N391D and D694N have also been constructed. The mutants N298D and N391D have been expressed in yeast and tested for thermal stability. The N298D enzyme shows increased thermostability as

compared to the wild type. This demonstrates that other modifications to increase thermal stability identified in Table 1 will also be effective.